Effectiveness of an Innovative Prototype Subtracted Diversity Array (SDA) for Fingerprinting Plant Species of Medicinal Importance

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Key words
- genotyping
- microarray
- diversity
- phylogenetics
- angiosperms

Abstract

The accurate identification of medicinal plants is becoming increasingly important due to reported concerns about purity, quality and safety. The previously developed prototype subtracted diversity array (SDA) had been validated for the ability to distinguish clade-level targets in a phylogenetically accurate manner. This study represents the rigorous investigation of the SDA for genotyping capabilities, including the genotyping of plant species not included during the construction of the SDA, as well as to lower classification levels including family and species. The results show that the SDA, in its current form, has the ability to accurately genotype species not included during SDA development to clade level. Additionally, for those species that were included during SDA development, genotyping is successful to the family level, and to the species level with minor exceptions. Twenty polymorphic SDA features were sequenced in a first attempt to characterize the polymorphic DNA between species, which showed that transposon-like sequences may be valuable as polymorphic features to differentiate angiosperm families and species. Future refinements of the SDA to allow more sensitive genotyping are discussed with the overall goal of accurate medicinal plant identification in mind.

Abbreviations

A:	angiosperm
AFLP:	amplified fragment length polymorphism
cDNA:	complementary DNA
DarT:	diversity array technology
DNA:	deoxyribonucleic Acid
gDNA:	genomic DNA
NA:	non-angiosperm
PCR:	polymerase chain reaction
RAPD:	random amplified polymorphic DNA
RFLP:	restriction fragment length polymorphism
SDA:	subtracted diversity array
SSH:	subtractive suppression hybridization
SSR:	simple sequence repeat

received July 21, 2008
revised January 30, 2009
accepted February 10, 2009

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DOI 10.1055/s-0029-1185484
Published online March 18, 2009
Planta Med 2009; 75: 1180–1185 © Georg Thieme Verlag KG Stuttgart · New York · ISSN 0032-0943

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Introduction

Medicinal plants are a valuable source for traditional and modern medicine and, during the past decade, demands for medicinal plants and their products have increased worldwide. Consequently, accurate identification of medicinal plants has become essentially important because of the reported concerns about purity, quality and safety [1]. Use of authentic herbal material is the first step to ensure quality, safety and efficacy of herbal medicines. The current regulatory guidelines suggest macroscopic and/microscopic evaluation or chemical fingerprinting for quality control and standardization of medicinal herbs [2]. However, incorrect identification is prevalent owing to limitations of these techniques [3,4], thus there is a strong need for a novel approach that can serve as an alterative to, or complement, these techniques.

Identification of plants using DNA fingerprinting, or genotyping, involves characterization of an organism based upon its unique DNA profile. The method offers a definitive means of authentication and has several advantages including; uniqueness for each taxon, identical results across different organs and physiological states, and only a small amount of tissue required [5]. "Fingerprinting" the genetic make-up of plants is extremely useful in many disciplines of plant science including the study of genetic variation, cultivar identification, phylogenetic analysis, geno-

typing, and in recent times for authentication and quality control of plant species of medicinal importance [6–9]. There exist many DNA-based techniques for fingerprinting, which are well reviewed in [10]. The practicality of each of these techniques varies in terms of their reliability, reproducibility, complexity, setting-up cost, operation cost, and throughput [11, 12]. The array-based methods are becoming much appreciated as modern techniques for genotyping [7, 12–17]. In particular, they offer a fast, economical, solid state technology for sequence-independent genotyping [11, 16, 18]. DNA polymorphisms at several hundred genomic loci can be detected in a single hybridization-based assay that distinguishes the presence versus absence of individual fragments in genomic representations, as demonstrated in a number of other studies [12, 15]. However, the effectiveness of this technique for genotyping purposes depends on the generation of sufficient amounts of polymorphic genomic DNA fragments (gDNA) from often complex plant genomes. The highly repetitive DNA content of plant genomes may result in cross-hybridization of many different genomic fragments, which can mask the potential polymorphisms and result in the conversion of polymorphic probes into monomorphic probes [11]. This becomes very critical when generating species or subspecies level fingerprints where genetic variation is much lower (low resolution) compared to the higher genetic variability that exits at the clade, order or family levels.

As an improvement to current microarray-based genotyping methods, Jayasinghe et al. [3] recently reported the construction of an innovative prototype subtracted diversity array (SDA) in which an alternative SSH was employed. The alternative SSH involved the pooling and SSH of gDNA representations from angiosperms and non-angiosperms to isolate gDNA sequences specific to flowering plants, thus potentially enabling the genotyping of a wide range of species. This technique has the power to produce more useful genotyping information without the inclusion of large numbers of array features, as well as eliminating the need for a discovery array (DArT) and pairwise SSHs (diversity SSH). However, the previous SDA pilot study [3] was executed only as a proof of concept so its application for a particular genotyping purpose was not investigated. This study reports the rigorous validation of the prototype SDA for the determination of its effectiveness for genotyping medicinal plants.

Materials and Methods

Development of SDA genome representations and library construction

The SDA used in this study was prepared as previously described [3]. The method involved selection of 49 angiosperm (including 46 medicinal herbs) and five non-angiosperm species were sourced to represent all clades within the two divisions. Only verified specimens of each species were collected, from which total DNA was extracted from fresh leaves using the Qiagen™ DNeasy® Plant Mini Kit (Qiagen) according to the manufacturer's guidelines. To develop genome representations the DNA samples were pooled into two main groups representing angiosperms (*A*) and non-angiosperms (*NA*), and 4 µg of DNA from each group were double digested at 37 °C for 6 h with 70 U of each restriction enzyme, *Alu*I and *Hae*III (Promega), in a total volume of 50 µL. As previously described [3], the *A* and *NA* digested DNA pools were used for the construction of a subtracted DNA library, which involved a subtractive hybridization technique between the two DNA populations to isolate the *A*-specific DNA [3]. Briefly, the Clontech PCR-Select™ cDNA Subtraction Kit (Clontech) was used according to the manufacturer's guidelines, but the protocol was modified to account for the double-stranded tester and double-stranded driver representing digested blunt-ended DNA fragments of *A* and *NA*, respectively. After the subtraction of *NA* fragments by suppression PCR, the *A*-specific fragments were cloned using the pGEM®-T Easy vector (Promega) and transformed into *Escherichia coli* JM109 competent cells (Promega), resulting in a subtracted DNA library of 376 clones with inserts of 250–1000 bp.

SDA fragment amplification and printing

The inserts of the 376 subtracted DNA library clones were amplified in 100 µL PCR reactions as previously described [3]. PCR products were transferred to V-bottom polypropylene 96-well plates and purified by ethanol/sodium acetate precipitation before resuspension in 10 µL of 50% DMSO (denaturing buffer). Four negative controls to be included on the SDA included buffer DMSO (50%), nested PCR primers 1 and 2R (Clontech) and pGEM®-T Easy Vector (Promega) double-digested with *Alu*I & *Hae*III. All controls were purified and resuspended in DMSO buffer as above and, together with the experimental DNA samples, were transferred to a 384-well microarray plate (Genetix). The SDA was printed using a BioRobotics® MicroGrid II Compact (Genomic Solutions) at RMIT University, Australia. Each sub-array was prepared with 384 samples (376 experimental DNAs and 8 controls) in a 48 × 8 format, where each element was deposited once with a volume of approximately 6 nL and diameter of 200 µm. Corning® GAPS II coated slides (Corning Incorporated Life Sciences) were used as the solid substrate on which six sub-arrays were printed per slide.

Target synthesis and SDA hybridization

Having already validated the SDA for clade-level genotyping of the angiosperm species (in-put groups) used to construct the SDA in a previous study [3], the ability to genotype species not included (out-put groups) for initial SDA construction to clade level was determined in this study. Out-put groups representing the Rosids, Asterids and Monocots (**Table 1**) were pooled as separate targets and genotyped with the SDA. To explore the ability of the SDA to genotype in-put angiosperm groups to family level within these three clades, pooled family targets were prepared according to **Table 1**, and hybridized to the SDA. Additionally, individual species targets within these families (**Table 1**) were also assayed to determine species level genotyping capability. The preparation of targets in all cases involved the double-digestion of 4 µg pooled total DNA with *Alu*I and *Hae*III and purification as described for the development of genome representations. Biotin-11-dUTP was then incorporated into newly synthesized complementary DNA strands using the Biotin DecaLabel™ DNA Labeling Kit (Fermentas) following the manufacturer's guidelines.

The SDA slides were pre-hybridized for 45 min at 42 °C in a pre-warmed solution containing 5 × SSC, 0.1% SDS, 1% BSA and 25% formamide. The slides were rinsed twice with sterile MilliQ water and immediately dried with an air gun. The Biotin-labeled targets (dried to 6.5 µL) were added to 12 µL of fresh 2 × hybridization buffer (500 µL formamide, 500 µL 10 × SSC, 20 µL 10% SDS), 2 µL of 5 µg/µL Cot1 DNA (Sigma-Aldrich), 1.5 µL of 10 mg/mL Poly A (Sigma-Aldrich) and 2 µL of 10 mg/mL salmon sperm DNA (Sigma-Aldrich). The mixture was denatured at 100 °C for 2 min and immediately applied onto two sub-arrays under a 22 × 25 mm

Table 1 List of the species pooled into representative angiosperm clade and family targets based upon known classifications [22]. Individual species targets assayed are indicated with an asterisk.

Target	Description	Representative/s
Clade in-put groups	Eumagnollids	*Magnolia denudata, Schizandra chinensis*
	Caryophyllids	*Dianthus superbus*
	Rosids	*Abutilon avicennae, Agrimonia pilosa, Astragalus membranaceus, Casuarina equisetifolia, Cicer arietinum, Dichroa febrifuga, Fragaria ananassa, Glycyrrhiza glabra, Isatis indigotica, Sedum sarmentosum*
	Monocots	*Allium cepa, Belamcanda chinensis, Bletilla striata, Coix lachryma-jobi, Fritillaria thunbergii*
	Ranunculids	*Clematis hexapetala*
	Asterids	*Angelica dahurica, Digitalis purpurea, Forsythia suspense, Leonurus sibiricus, Lycium barbarum, Perilla frutescens, Physalis alkekengi, Platycodon grandiflorus, Salvia miltiorrhiza*
Clade out-put groups	Rosids	*Rosa chinensis, Geranium atropurpureum*
	Monocots	*Zea mays, Arisaema triphyllum*
	Asterids	*Aquilegia flavescens, Petroselinum crispum*
Family groups	Rosaceae	*Agrimonia pilosa*, Fragaria ananassa*
	Fabaceae	*Astragalus membranaceus, Glycyrrhiza glabra*, Glycyrrhiza uralensis**
	Lamiaceae	*Leonurus sibiricus*, Salvia miltiorrhiza, Perilla frutescens*
	Solanaceae	*Lycium barbarum, Physalis alkekengi**
	Iridaceae	*Belamcanda chinensis**
	Liliaceae	*Fritillaria thunbergii*, Allium cepa**
	Poaceae	*Coix lachryma-jobi**

lifter slip (Grale Scientific). Slides were then placed in waterproof, humidified hybridization chambers (Corning Incorporated Life Sciences) and incubated overnight in a 50 °C water bath. Following hybridization, slides were washed for 5 min in 2 × SSC with 0.1% SDS, 5 min in 0.5 × SSC with 0.1% SDS, 5 min in 2 × SSC, 5 min in 4 × SSC with 0.2% Tween 20 and rinsed with sterile MilliQ water. The biotinylated DNA targets bound on the array were then labeled with fluorescent FluoroLink™ streptavidin-labeled Cy™3 dye (Amersham Pharmacia) using a biotin-streptavidin system. Briefly, 24 µL of a 1:250 streptavidin-Cy™3:MilliQ water dilution were applied directly onto the array surface under a 22 × 25 mm lifter slip (Grale Scientific), and slides were placed in hybridization chambers and incubated for one hour in the dark. Finally, slides were washed in 4 × SSC with 0.2% Tween 20 for 2 s, 5 min in 0.1% SSC and rinsed with sterile MilliQ water before being dried with an air gun. All hybridizations were performed with two technical replicates (corresponding to two sub-arrays) and two biological replicates, resulting in four data points for each array feature.

Data analysis

Sub-arrays of each slide were scanned separately at 532 nm (Cy3 green laser) and 10 µm resolution using an Affymetrix® 428™ array scanner, and captured with the Affymetrix® Jaguar™ software (v. 2.0). Image analysis was performed using Imagene™ v. 5.5 (BioDiscovery) image analysis software. Spots were individually quantified using the fixed circle method; sample values were measured as the mean of pixels within the spot circle and the local background in a five-pixel diameter ring that began five pixels outside the spot circle. Automatic flagging eliminated empty spots, negative spots (signal mean < background mean), and poor spots (contaminated background, ignored pixels > 25%, open perimeter > 25%, offset from expected position > 60%). Quantified spot data was then exported to Microsoft Excel (Microsoft) and, for each "good" spot that passed all quality control criteria, a signal : background mean ratio was calculated. Spots with a signal : background ratio > 2.0 were considered to represent positive spots, and their values were converted to 1, whilst spots with a signal : background ratio < 2.0 were converted to 0. Only those features with "good" spots of consistent classification (positive or negative) in all four replicates of each hybridization were accepted for data analysis. Subsequently, the four replicates for each spot were combined as either a positive (1) or negative (0) spot. Using SPSS v. 13.0 (SPSS, Inc.), the resulting binary data were examined for phylogenetic relationships by constructing a dissimilarity dendrogram using Pearson correlation and hierarchical cluster analysis with between-groups linkage.

Sequence characterization of SDA features

Twenty randomly selected positive SDA features were sequenced from cloned plasmids of the original gDNA library [3]. All clones were subjected to single-pass sequencing from the 5′ end of the vector using BigDye™ Terminator Chemistry (PE Biosystems) and an ABI Prism 377 DNA Sequencer (Microbiology Department, Monash University, Victoria, Australia, and Macrogen Company, World Meridian Venture Centre, Gasan-dong, Geumcheon-Gu). Vector sequences were manually removed from sequence reads, which were characterized using blastn and blastx (National Centre for Biotechnology Information; http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) to determine sequence homology with existing entries in the non-redundant nucleotide and protein databases. Database hits were ranked by expectation (e) value, and were regarded as significantly similar to the input sequence if < 1e-10.

Results and Discussion

This study investigated the effectiveness of the SDA for genotyping species not included in the initial angiosperm pool used for its construction, as well as to lower classification levels. Firstly, we determined effectiveness of the SDA for differentiating individuals outside the initial angiosperm pool (out-put groups) into their respective clades. For this, we hybridized the SDA with clade-specific targets representing the Rosids, Asterids and Monocots. These targets were prepared by pooling gDNA of individual species outside the initial angiosperm pool into their known clades (**Table 1**). The hybridization patterns were used to construct a

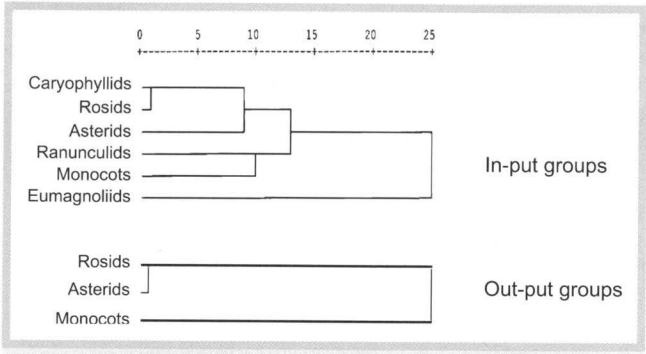

Fig. 1 Dissimilarity dendrogram (Pearson correlation, between-groups linkage) for the SDA hybridization patterns of the six angiosperm clades for in-put groups [3], compared to the dendrogram obtained for angiosperm clades of the out-put groups. The steps of the dendrogram show the combined clusters and the values of the distance coefficients at each step; the values have been rescaled to numbers between 0 and 25, preserving the ratio of the distances between the steps.

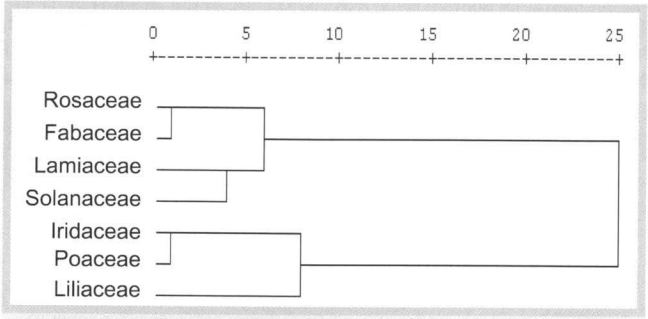

Fig. 2 Dissimilarity dendrogram (Pearson correlation, between-groups linkage) for the SDA hybridization patterns of the seven in-put angiosperm families within the Rosids (Rosaceae and Fabaceae), Asterids (Lamiaceae and Solanaceae) and Monocots (Iridaceae, Poaceae and Liliaceae) clades. The steps of the dendrogram show the combined clusters and the values of the distance coefficients at each step; the values have been rescaled to numbers between 0 and 25, preserving the ratio of the distances between the steps.

hierarchical cluster dendrogram using Pearson correlation and between-groups linkage (**Fig. 1**). As we previously observed for clade-level analysis of the in-put groups [3], the Rosids and Asterids were closely related while Monocots are more distant (**Fig. 1**). This result indicates that the SDA could be used to differentiate not only in-put groups to clade level, but other species outside the initial angiosperm pool. For this clade-level analysis, we identified 309 good quality SDA features, of which 112 were common to both Rosids and Asterids but not found in Monocots. Additionally, there were four Rosid-specific features and one Monocot-specific feature. Interestingly, the Rosid- and Monocot-specific features identified in our previous study of in-put groups were not the same as those identified for this out-put groups analysis. In particular, two of the four Rosid-specific sequences detected for out-put groups were not detected with the in-put groups. The one Monocot-specific sequence obtained for out-put groups was not detected with the in-put groups. These differences could have been either due to a "dilution effect", where low-frequency sequences remain undetected within complex targets; gDNA from 10 individuals were pooled for the in-put Rosid target, while gDNA from two individuals were pooled for the out-put Rosid target. Alternatively, the differences may arise from the stringent data analysis applied to identify good quality features; for in-put groups there were 172 good features, while there were 309 good features for out-put groups.

To explore the discriminatory power of the SDA we chose to dissect three clades; Rosids, Asterids and Monocots. After SDA analysis of targets representing seven families within these clades (**Table 1**), hierarchical cluster analysis was performed as previously described (**Fig. 2**). At this level, the Rosid families (Rosaceae and Fabaceae) grouped together, as did the Asterid (Lamiaceae and Solanaceae) and Monocot families (Iridaceae, Poaceae and Liliaceae). Importantly, the family level dendrogram retained the overall clade level clustering pattern. Subsequently, these results showed that the SDA was able to effectively classify different families within these three clades. Our next goal was to test the effectiveness of the SDA for differentiating different species within these families. After hybridizing the SDA with species-specific targets and constructing a dissimilarity dendrogram (**Fig. 3**), we found that the majority of the assayed species were grouped with their corresponding families. However, *F. thunbergii* was clustered with Asterid species when it was expected to be clustered with Monocot species. Most probably, this result may be due to the very large genome (~38000 Mbp) of *F. thunbergii* [19]. Even with subtraction, the SDA of 384 features may not have included a sufficient proportion of the *F. thunbergii* genome to facilitate its identification. The two individual species, *G. glabra* and *G. uralensis*, although grouped with other Rosid species, were clustered further apart than expected. This result suggests either that the SDA features were not sufficient enough to differentiate very closely related individuals, or the stringent data analysis employed for this study may have eliminated some critical information from the data set. We believe that latter would be the most probable reason as subsequent analysis of the data with alternative (less stringent) techniques resulted in the establishment of closer relationship between *G. glabra* and *G.uralensis* (data not shown). Overall, the ability of the SDA to genotype to species level requires some improvements, including an expansion of the number of SDA features as well as refinement of the stringent data quality control.

As a first attempt to characterize positive SDA features, 20 randomly selected features were sequenced (**Table 2**). Annotation of the DNA sequences showed that only three features included known genes; photosystem I P700 chlorophyll A apoprotein, NADH dehydrogenase subunit 2 and ATP synthase beta subunit. Seven features were annotated as encoding predicted or uncharacterized proteins, two features matched open reading frames (ORFs), and one feature obtained no hit in the databases. Interestingly, the remaining seven features represented transposon-like sequences, which may explain the hybridization specificity observed for these features. Among these seven transposon-like sequences, four showed specific family-level polymorphism within the angiosperms (**Table 2**). This represents a high proportion considering that only 10 of the total 20 sequences showed such polymorphism, and the remaining 10 hybridized to all angiosperm families. Transposons constitute a major proportion of the intergenic regions of higher plant genomes, and have a significant impact on the evolution of gene control and genome structure [20]. Transposons, as mobile genetic elements, cause mutation and DNA variation across a genome, thus could be expected to be a source of polymorphism for DNA fingerprinting. In rice, a recent example of using transposons for DNA fingerprinting

Table 2 Functional characterization of the 20 sequenced subtracted diversity array (SDA) features using blastx (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/blast/Blast.cgi), showing the best matching NCBI protein database entry, e value (regarded as significant if < 1e-10), and putative functional description for each sequence. NA indicates the absence of significant data.

SDA feature	Specific to target	Matching database entry	e value	Putative function
A009	Angiosperms	Q6U1F5	2e-79	photosystem I P700 chlorophyll A apoprotein
A014	Angiosperms	A4QMB1	8e-21	ORF58e
A017	Asterids	Q0KIP3	8e-32	polyprotein, 3'-partial, putative
A018	Monocots	Q58MG0	6.2	putative uncharacterized protein
A028	Angiosperms	A7T7N0	3e-13	predicted protein
A043	Monocots	A7SHI0	0.15	predicted protein
A060	Angiosperms	A8NMG1	6e-17	putative uncharacterized protein
A138	Asterids		NA	no hits
A145	Asterids, Caryophyllids, Ranunculids, Rosids and Monocots	Q05H69	1e-09	NADH dehydrogenase subunit 2
A187	Angiosperms	Q3HKA5	2e-22	putative uncharacterized protein
B016	Asterids, Eumagnoliids, Rosids and Monocots	Q2R2I2	5e-23	retrotransposon protein, putative, Ty1-copia subclass
B030	Angiosperms	O23864	5e-74	polyprotein
B037	Angiosperms	A4GYV9	3e-26	putative uncharacterized protein
B077	Angiosperms	Q8W5J8	1e-05	putative retroelement
B113	Rosids	Q0KIP3	2e-26	polyprotein, 3'-partial, putative
B133	Angiosperms	A4QMB0	2e-29	ORF64d
B151	Asterids, Ranunculids and Monocots	Q9TLJ2	5e-36	ATP synthase subunit beta
B190	Rosids and Monocots	B1N668	3e-29	copia LTR rider
B240	Angiosperms	Q8S8M1	5e – 20	putative retroelement integrase
B266	Monocots	Q2TYZ8	0.56	predicted protein

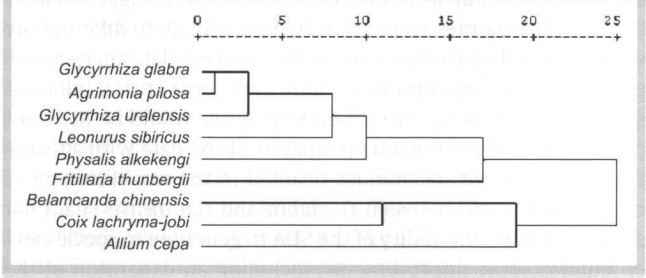

Fig. 3 Dissimilarity dendrogram (Pearson correlation, between-groups linkage) for the SDA hybridization patterns of the nine in-put angiosperm species within the Rosaceae (*A. pilosa*), Fabaceae (*G. glabra* and *G. uralensis*), Lamiaceae (*L. sibiricus*), Solanaceae (*P. alkekengi*), Iridaceae (*B. chinensis*), Poaceae (*C. lachrymal-jobi*), and Liliaceae (*F. thunbergii* and *A. cepa*) families. The steps of the dendrogram show the combined clusters and the values of the distance coefficients at each step; the values have been rescaled to numbers between 0 and 25, preserving the ratio of the distances between the step.

found that a transposon-derived molecular marker was able to distinguish between cultivated rice species [21]. The results of the present study that show a higher level of family-level polymorphism for the transposon-like sequences indicate that many of the polymorphic SDA features may include transposon-like sequences, which could be used as family- and species-specific probes in future studies. To this end, the sequencing of more polymorphic SDA features will be a focus of future studies.

The long-term goal of this study is to develop an SDA that is effective for fingerprinting plant species of medicinal importance for their authentication. Bearing in mind that this is only a prototype SDA, the results thus far are highly promising. The prototype SDA is effective for genotyping both in-put and out-put groups at clade level, and is also capable of further differentiation of clades into their corresponding families, and families to their corresponding species. However, for the correct identification of some species (e.g., species with larger genomes and for closely related species), the prototype SDA needs to undergo further improvements. Incorporation of more representatives for the initial angiosperm and non-angiosperm pools will facilitate more efficient subtraction. Development of a larger SDA with more features will not only facilitate a wider range of species identification but will also increase the discriminatory power. Modifications to the SDA design, for example, development of clade-specific panels rather than one angiosperm-specific panel may also help to increase the discriminatory power without the inclusion of a larger number of additional array features. The highly stringent data analysis seems to eliminate some useful information from the data sets and, although not a problem for establishing relationships at higher classification levels, it limits the establishment of relationships between species where differences are small. Thus far, we have explored the effectiveness of SDA technique for fingerprinting only fresh medicinal plant species. Considering the frequent occurrence of medicinal plant materials in dried form, it would also be useful to know if this technique could be equally suitable for genotyping dried plant tissue, which will be studied in the future.

Acknowledgements

The authors wish to acknowledge the funding of this research by an RMIT University VRII grant.

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